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# Impact of Exogenous Sequences on the Characteristics of an Epidemic Type 2 Recombinant Vaccine-Derived Poliovirus[▽]

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**Pathogenic circulating vaccine-derived polioviruses (cVDPVs) have become a major obstacle to the successful completion of the global polio eradication program. Most cVDPVs are recombinant between the oral poliovirus vaccine (OPV) and human enterovirus species C (HEV-C). To study the role of HEV-C sequences in the phenotype of cVDPVs, we generated a series of recombinants between a Madagascar cVDPV isolate and its parental OPV type 2 strain. Results indicated that the HEV-C sequences present in this cVDPV contribute to its characteristics, including pathogenicity, suggesting that interspecific recombination contributes to the phenotypic biodiversity of polioviruses and may favor the emergence of cVDPVs.**

Poliovirus (PV), the causative agent of acute paralytic poliomyelitis, is a single-stranded RNA human enterovirus (HEV). Albert Sabin's trivalent live-attenuated oral PV vaccine (OPV) was licensed in the 1960s and has now become the main tool of the wild-type-PV eradication program set up by the World Health Organization (WHO) in 1988 (http://www.polioeradication.org/). However, reversion to neurovirulence of OPV strains has since been observed in both immunodeficient (20, 34) and healthy (21) individuals. Indeed, the genetic instability of OPV strains (13, 23) due to RNA-dependent RNA polymerase error rate and recombination coupled with selective pressure may have contributed to the emergence of pathogenic vaccine-derived PVs (VDPVs).

Recent poliomyelitis outbreaks due to circulating VDPVs (cVDPVs) have brought to the forefront the depth of enterovirus evolution and the influence it may have on the eradication program (14). cVDPV outbreaks occurred subsequently to a period of low vaccine coverage in nine different countries, including Madagascar (8, 12, 17, 31, 32, 37–39). All cVDPV outbreaks but one were due to recombinants originating from OPV strains; large parts of the genomic regions encoding the nonstructural proteins in these strains and the 3′ untranslated region (UTR), and in some cases the 5′ UTR, were derived from HEV species C (HEV-C) (8, 12, 17, 31, 32, 37–39), enteroviruses that are phylogenetically closely related to PVs (4). Genetic analyses of recombinant cVDPV strains and cocirculating enteroviruses have recently shown that HEV-C isolates (coxsackievirus strains A13 and A17) exhibit highest similarity with these cVDPVs in the genomic regions encoding the nonstructural proteins and in the 3′ UTR (3, 26).

Recombination is a common evolution mechanism for enteroviruses that is thought to contribute at least to the repair of deleterious mutations in genomes (1). However, the role of interspecific genetic exchanges in the evolution and functions of OPV/HEV-C recombinants is still unclear. Two type 2 recombinant cVDPV lineages with sequences from the OPV type 2 strain in the 5′ half of the genomes and with sequences derived from HEV-C in the 3′ half have been isolated from children with poliomyelitis in Madagascar in 2002 (31). These cVDPV strains showed phenotypic characteristics similar to those of wild-type neurovirulent viruses, including neurovirulence in PV receptor-transgenic (PVR-Tg) mice (26). To investigate the impact of genetic exchanges between PV and HEV-C species on the characteristics of recombinant viruses, we generated and characterized chimeric viruses with various crossover regions between one type 2 Madagascan cVDPV isolate (MAD04) and the original Sabin OPV type 2 strain (Sabin2).

**Nomenclature for viruses.** Natural viruses were Sabin2 (WHO laboratory stock of Sabin2) and MAD04 (accession number AM084223). The viral stock used in this experiment was obtained following the amplification of a plaque-purified virus derived from the original MAD04 isolate described in reference 26. Although this plaque-purified virus differs from the original isolate by a few nucleotides (nt) (nt 704 C→T, 949 C→T, 3576 T→C, 5334 A→G, 6897 C→T, 6910 T→C [Sabin2 sequence numbering]), it exhibited similar phenotypic characteristics.

Viruses derived from cDNA (MAD04 series) were strains cS2 and cMAD04 for Sabin2 and MAD04, respectively (parental), and strains cS2/MAD04, cMAD04/S2, and cMAD042A/S2 (chimeric).

**Construction of cDNA-derived viruses.** Taking into account the natural recombination site between the vaccine-derived part and the nonvaccine (HEV-C) part of the MAD04 genome in the protease 2A coding region (nearby nt 3801), infectious viral cDNAs were cloned and three recombinants were constructed (Fig. 1). Briefly, PCR amplifications of four half-viral genomes (from Sabin2 and MAD04) were performed using (i) MAD04 viral RNA extracted by the Trizol reagent (Invitrogen) from MAD04 viral particles purified by ultracentrifugation or plasmid pT7S2 (a plasmid harboring the whole Sabin2 cDNA; generous gift of A. Macadam) as a template, (ii) specific primers flanked with either an AscI restriction site and the

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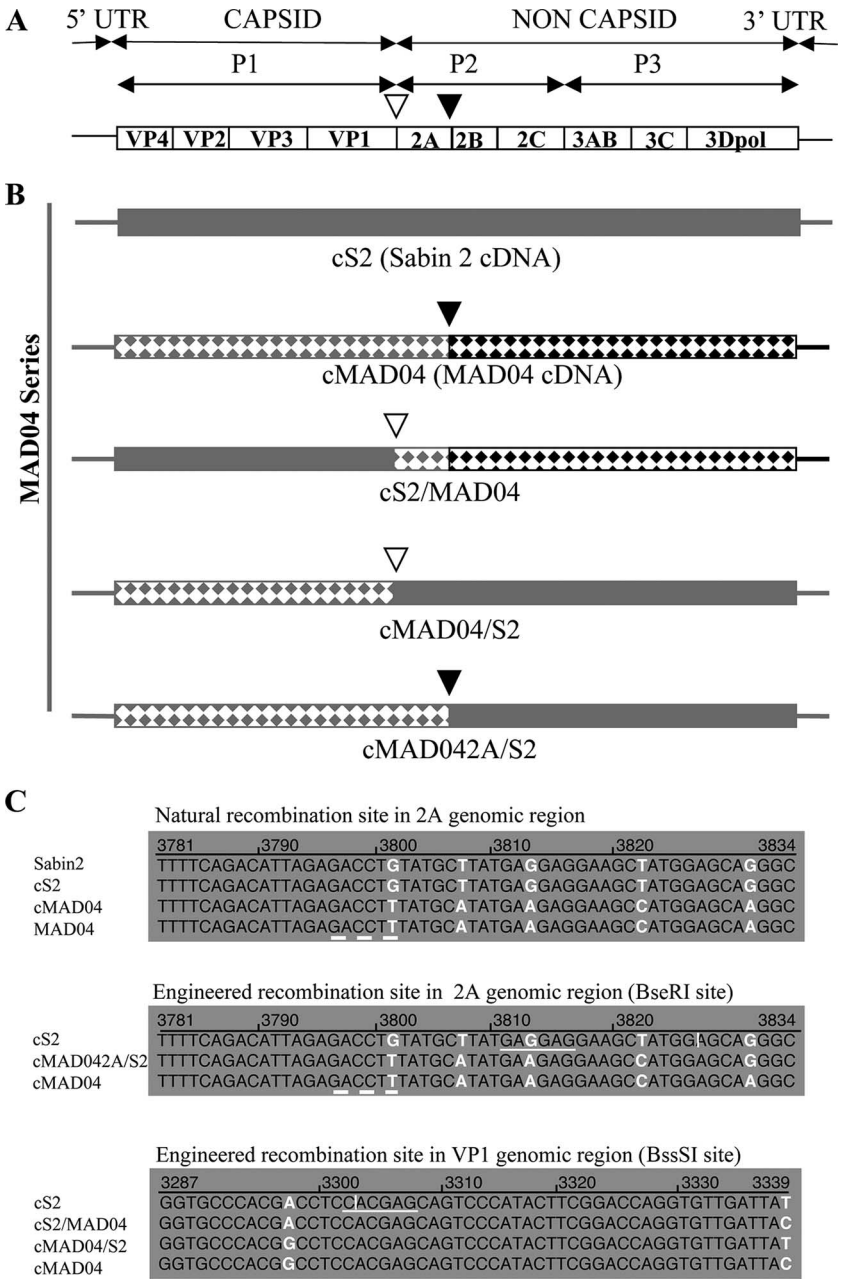


FIG. 1. Genomic structure of in vitro-generated parental and chimeric viruses. (A) The genetic organization of the PV genome is shown (top row), including the 5′ and 3′ UTRs. Genomic regions encoding viral proteins (VP4 to 3Dpol) and their precursors P1 to P3 are indicated. (B) The cDNA-derived parental and chimeric viruses of the MAD04 series are shown. The cDNA-derived Sabin2 (cS2) and MAD04 viruses (cMAD04) are presented. The mutated vaccine-derived part (the 5′ half) and the HEV-C part (the 3′ half) of the cMAD04 genome are indicated together with the natural recombinant site (filled triangles). This natural site and another intentionally determined site located at the boundary between the capsid and noncapsid genomic regions (open triangles) were used for constructing chimeric cDNAs (cS2/MAD04, cMAD04/S2, and cMAD042A/S2). (C) Nucleotide sequence alignments showing the natural and engineered recombination sites. Nucleotide numbering is indicated according to the Sabin2 genome. Nucleotide positions of the natural recombination site in the MAD04 isolate (approximate position) (white dotted line) and of the recognition sequences of the restriction enzymes used for engineering chimeric cDNAs (horizontal white solid lines) and their respective cleavage sites (white vertical lines) are given. Nucleotides differentiating cS2 from cMAD04 are in white.

T7 RNApol promoter sequence at the 5′ end or a NotI restriction site at the 3′ end of the genome, and (iii) oligonucleotide primers on either side of a BssSI restriction site (nt 3303; 3′ end of the VP1 region, codon 274). Fragments were then TA cloned into the pGEM-T vector (Promega) and fully sequenced. No differences in the 5′ UTR and 3′ UTR sequences or in the viral polypeptides inferred from the coding regions between natural and cDNA-derived viruses were observed. The half-viral genomes were then sequentially subcloned into a modified pBR322 vector containing a custom-made cloning

cassette (HindIII-AscI-BssSI-NotI-EcoRI). Using the BssSI restriction site, we generated the parental full-length cDNAs, cS2 and cMAD04, and the chimeric ones, cS2/MAD04 and cMAD04/S2 (Fig. 1). The chimeric cDNA cMAD042A/S2 (recombination site, nt 3827; protease 2A codon 148) was generated by replacing in cMAD04/S2 a BssSI-BseRI restriction fragment (nt positions 3,303 to 3,827) amplified from cMAD04. Recombination did not modify the whole VP1 and 2A polypeptide sequences found in MAD04 and Sabin2. Constructs and mutations were verified by DNA sequencing.

The T7 RNApol promoter, present upstream from the cloned viral cDNAs, was used to transcribe viral RNAs from linearized plasmids (T7 RiboMax; Promega). RNA transcripts were transfected by DEAE-dextran into HEp-2c cell monolayers (36) and incubated at 37°C. Results of three independent experiments gave an average infectivity rate of 2.0 ($\pm$ 0.7) $\times$ $10^4$ PFU/$\mu$g of RNA for all viruses, indicating that the essential functions of the constructed chimeric viruses were not impaired in comparison to their cDNA-derived parental viruses. Viral stocks of MAD04 series viruses were obtained following the transfection of 1 $\mu$g of RNA/$10^6$ cells in liquid media and two subsequent passages in HEp-2c cells.

**Phenotypic characteristics of chimeric viruses.** Phenotypic characteristics of parental cDNA-derived viruses were compared to those of natural viruses. No significant differences were observed. For each construct used, two different cDNA-derived viruses obtained from separate cDNA clones were tested.

Replication kinetics of natural and MAD04 series viruses were compared in HEp-2c cells (6). For single-step growth experiments, cells were infected at a multiplicity of infection (MOI) of 25 50% tissue culture infectious doses ($TCID_{50}$) per cell and incubated at 37°C, and viral titers ($TCID_{50}$/ml) were determined at various time points postinfection (6) (Fig. 2A and B). Overall, virus replication kinetics between natural and cDNA-derived viruses (Fig. 2A) are very similar, and growth curves of chimeric viruses were rather similar to those of both parental viruses (Fig. 2B). Moreover, results using a low MOI (1 $TCID_{50}$ per cell) do not indicate major differences between all viruses (Fig. 2C). Although the MAD04 virus appears to replicate slightly better than the other viruses (Fig. 2B) and the growth of cMAD04/S2 and cMAD042A/S2 appeared slightly delayed compared to cS2, cS2/MAD04, and cMAD04 (Fig. 2C), the differences do not appear to be significant according to longitudinal data analysis. These results indicated that recombination exerts only a mild effect or no effect at all on viral growth in HEp-2c cells.

Characterization of MAD04 series viruses was then achieved by assaying plaque size and temperature sensitivity in HEp-2c cells as described previously (26) (Table 1). The plaque size of cMAD04 was 3.5-fold larger than that of cS2 ($P < 0.01$ [Kruskal-Wallis test]). The plaques of chimeric viruses cMAD042A/S2 and cS2/MAD04 exhibited intermediate sizes between those of cMAD04 and those of cS2 ($P < 0.01$). The plaque sizes of cMAD04/S2 appeared to be similar to those of cS2. Protease 2A from MAD04 that is present in cMAD042A/S2 and cS2/MAD04, differing from that of cS2 by a few amino acid (aa) residues (26), may be implicated in the intermediate-plaque-size phenotype.

Temperature sensitivities of viruses were evaluated by an Rct (reproductive capacity at different temperatures) test, the results of which are the differences between the $\log_{10}$ virus titer of a viral stock measured at the optimal temperature (37°C) and that measured at the supraoptimal temperature (40.2°C).

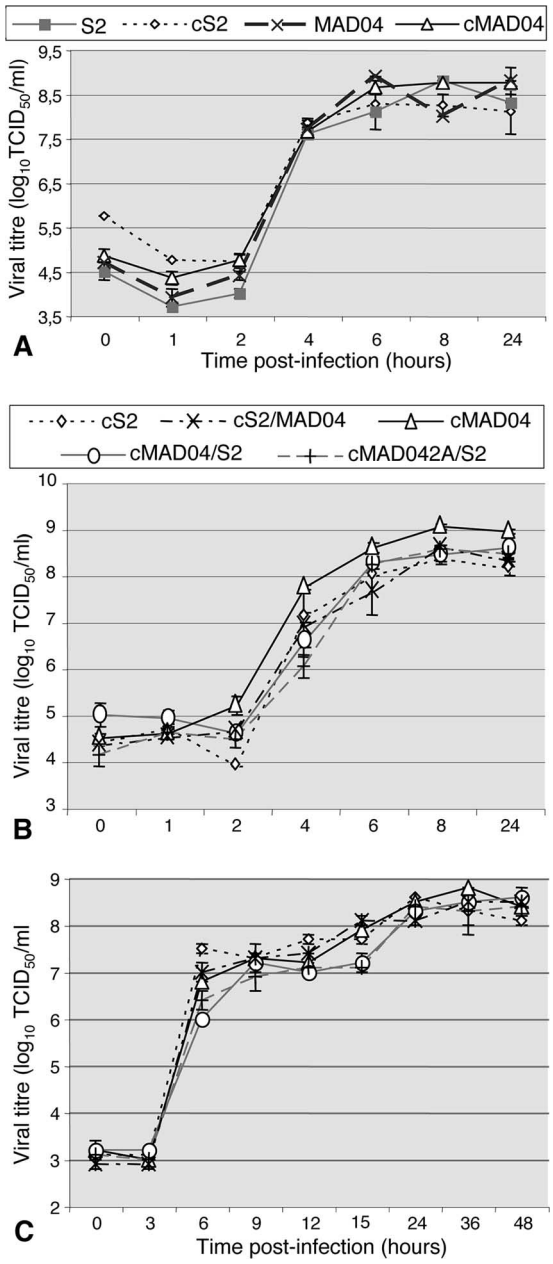


FIG. 2. Growth curves of parental and chimeric viruses. HEp-2c cells were infected with the indicated viruses, cells and supernatants were harvested at various time points, and each sample was titrated by a microtitration method. Standard errors from the means of different samples are shown as error bars. (A) Natural viruses (Sabin2 and MAD04) and parental cDNA-derived viruses (cS2 and cMAD04). (B and C) MAD04 series viruses. Whereas growth curves for panels A and B were obtained following infection of HEp-2C cells at a high MOI (25 $TCID_{50}$ per cell for single-step growth curves), curves for panel C were obtained following infection at a low MOI (1 $TCID_{50}$ per cell). (B) Mean values obtained from two different experiments using two batches of MAD04 series viruses derived from different clones of the same plasmidic chimeric genomic cDNA constructs.

TABLE 1. Phenotypic markers of the parental and chimeric PV strains

| Strain | Plaque size (mm)[a] | Temperature sensitivity ($log_{10}$ $TCID_{50}$/ml)[b] | PD50 ($log_{10}$ $TCID_{50}$/mouse)[c] |
|---|---|---|---|
| cS2 | 0.41 (0.11) | 5.75 (0.35) | >6.6 |
| cMAD04/S2 | 0.47 (0.14) | 3.33 (0.31) | 5.2 (0.3) |
| cMAD042A/S2 | 0.63 (0.17) | 3.25 (0.18) | 5.3 (0.3) |
| cS2/MAD04 | 0.65 (0.10) | 3.17 (0.22) | >6.6 |
| cMAD04 | 1.38 (0.50) | 1.33 (0.40) | 3.7 (0.3) |

[a] The mean plaque diameters are shown. The standard deviations are shown in parentheses.

[b] The differences between the $log_{10}$ virus titer of a viral stock measured after 7 days at the optimal temperature (37°C) and that measured at the supraoptimal temperature (40.2°C), determined by an Rct test, are shown. The data are arithmetic means from two experiments with standard errors of the means shown in parentheses.

[c] The paralytic doses affecting 50% of inoculated mice (PD50) are indicated. Groups of six mice (three males and three females) were inoculated intracerebrally with 40 μl of 10-fold dilutions of viral stocks. Similar results were obtained for two different stocks derived from separate recombinant cDNA clones of the same viruses. The viral doses that induced paralysis or death in 50% of mice after 21 days were calculated from cumulated data by the method of Reed and Muench (27), and the standard errors (data in parentheses) were determined by the formula of Pizzi (24). >6.6, no disease occurred following the inoculation of the highest doses.

Chimeric viruses harbored intermediate-temperature-sensitivity phenotypes between that of the temperature-sensitive parental cS2 and that of the non-temperature-sensitive cMAD04 (Table 1).

Phenotypic characterization of the cDNA-derived MAD04 series indicated that determinants located in both the vaccine part and the nonvaccine part of the MAD04 genome contribute to the temperature sensitivity and plaque size phenotypes and may slightly influence its replication capacity. However, we cannot exclude that these aspects result from cooperative functional interactions between both genomic parts and/or corresponding viral proteins.

**Neurovirulence of chimeric viruses.** The pathogenicity of MAD04 series viruses was first evaluated by determining the paralytic dose affecting 50% of the inoculated mice (Table 1). This was achieved by intracerebral inoculation of serial dilutions of viral stocks into PVR-Tg21 mice genetically modified to express the human PV receptor gene (generous gift from A. Nomoto; experiments were approved by and conducted in accordance with the guidelines of the Office of Laboratory Animal Care at the Institut Pasteur) (10, 26). Virus cMAD04 showed potent neurovirulence that was comparable to that of the virulent S2/4568, a highly neurovirulent type 2 VDPV isolated from environmental waters (33). In contrast, the cMAD04/S2 and cMAD042A/S2 viruses exhibited only moderate neurovirulence in PVR-Tg21 mice. Finally, the cS2/MAD04 virus did not induce paralysis in mice inoculated with the highest doses (6.6 $TCID_{50}$/mouse), thus showing an attenuated phenotype similar to that of cS2. Similar differences between neurovirulence levels were obtained when PVR-Tg21 mice were inoculated, via the intranasal (IN) and intraperitoneal routes (IP), with given amounts of parental and chimeric viruses (Fig. 3). Groups of mice inoculated with cMAD04/S2 and cMAD042A/S2 were significantly less affected than those that received S2/4568 and cMAD04 ($P < 0.01$ and $P < 0.05$ for mice inoculated via IN and IP routes, respectively [log rank test]).

These results indicated that the primary neurovirulence determinants of MAD04 are located within the 5′ half of the genome. Attenuation determinants of Sabin2 and of the attenuated related strain P2/P712 have been shown to be located in the 5′ UTR (nt 481) and in the VP1 genomic region (aa 143) (19, 28). The presence of these determinants in cS2/MAD04 (as well as in cS2) is likely to be one contributing factor rendering this chimeric strain nonpathogenic following intracerebral inoculation in adult PVR-Tg21 mice. The 5′ UTR determinant, implicated in the temperature sensitivity of the strain, is thought to affect the structure of the internal ribosomal entry site, the translation of the viral genome, virus multiplication in certain tissues and cells, including in neuronal cells, and virus spread in the infected host (18, 25). The VP1 determinant may affect conformation and/or stability of the loop of the virion surface in which it is located and those of neighboring loops (16). Nevertheless, some other molecular determinants and the possible interactions between them may contribute to the attenuated phenotypes of Sabin2 and cS2/MAD04 (29, 30). In fact, both attenuation determinants mentioned above were mutated in the neurovirulent cMAD04, cMAD04/S2, and cMAD042A/S2 viruses (nt 481 A→G and aa 143 Ile→Thr, respectively) as well as in previously described pathogenic Sabin2 mutants (5, 7, 19, 26, 28). However, our results indicated that the HEV-C-derived 3′ half of the MAD04 genome modulates pathogenicity maybe by slightly improving the capacity of the virus to replicate in certain tissues. Actually, it has

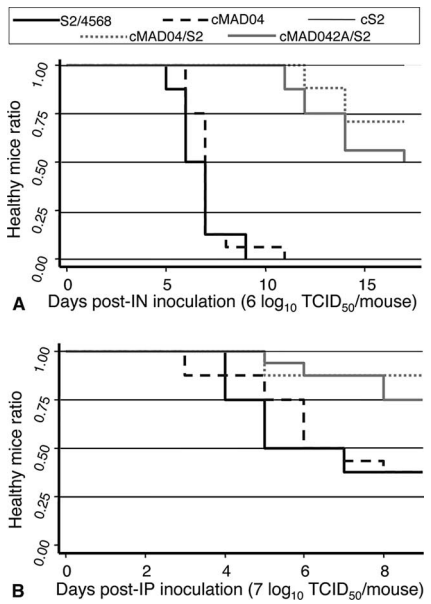


FIG. 3. Neurovirulence of MAD04 series viruses in Tg mice. A given dose of virus was inoculated via IN (A) or IP (B) routes in groups of PVR-Tg21 mice expressing the human PV receptor (6 to 16 animals per virus). Animals were checked daily for 21 days for the appearance of paralysis or death. Healthy-mouse ratios following inoculation of either parental or chimeric viruses or the neurovirulent positive-control virus S2/4568 (33) are represented. No additional paralyzed or dead mice were detected after day 17 (A) and day 9 (B) postinoculation.

been previously shown that the P3-3′ UTR regions of PV, coxsackievirus B3, and enterovirus 71 strains contribute to pathogenicity in animal models and/or to temperature sensitivity (2, 9, 22, 35). Comparative analysis of cMAD04/S2 and cMAD042A/S2 properties suggested that protease 2A (P2 region) plays only a minor role or no role at all in the pathogenicity and in other characteristics of MAD04, despite a few aa residues differing from those of Sabin2 (26).

**Impact of genetic exchanges on recombinant PV/HEV-C.** Recently, the characterization of recombinants between the highly pathogenic PV strain Mahoney and a prototype HEV-C (coxsackievirus A20) suggested that the P2 or P3 region of the PV genome can be exchanged with that of coxsackievirus A20 without any effect on viral multiplication or, at least in some cases, on pathogenicity for PVR-Tg mice (11). This suggests that recombination between PV and HEV-C can in some cases be neutral. Our experiments indicate that regardless of the evolution process following genetic recombination events, recombination between OPV strains and HEV-C brings together different genomic fragments that may contribute through their intrinsic properties and their interactions with the specific features of recombinant cVDPVs. The differences between our results and those mentioned above (11) can be explained by some parameters, including the nature of the PV genomes used in each study of either wild-type or vaccine origin and the HEV-C sequences used. Based on this study, recombination of PVs with other enteroviruses appeared to be not solely an indicator of intense circulation of the vaccine strains (15) but also an evolutionary process that may influence crucial viral properties, such as circulation and neurovirulence. Interspecific genetic recombination with HEV-C contributes to both genetic and phenotypic biodiversities of PVs and may be responsible, in part, for the emergence of cVDPVs.

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## REFERENCES

1. **Agol, V. I.** 2006. Molecular mechanisms of poliovirus variation and evolution. Curr. Top. Microbiol. Immunol. **299:**211–259.
2. **Arita, M., H. Shimizu, N. Nagata, Y. Ami, Y. Suzaki, T. Sata, T. Iwasaki, and T. Miyamura.** 2005. Temperature-sensitive mutants of enterovirus 71 show attenuation in cynomolgus monkeys. J. Gen. Virol. **86:**1391–1401.
3. **Arita, M., S. L. Zhu, H. Yoshida, T. Yoneyama, T. Miyamura, and H. Shimizu.** 2005. A Sabin 3-derived poliovirus recombinant contained a sequence homologous with indigenous human enterovirus species C in the viral polymerase coding region. J. Virol. **79:**12650–12657.
4. **Brown, B., M. S. Oberste, K. Maher, and M. A. Pallansch.** 2003. Complete genomic sequencing shows that polioviruses and members of human enterovirus species C are closely related in the noncapsid coding region. J. Virol. **77:**8973–8984.
5. **Cherkasova, E. A., M. L. Yakovenko, G. V. Rezapkin, E. A. Korotkova, O. E. Ivanova, T. P. Eremeeva, L. I. Krasnoproshina, N. I. Romanenkova, N. R. Rozaeva, L. Sirota, V. I. Agol, and K. M. Chumakov.** 2005. Spread of vaccine-derived poliovirus from a paralytic case in an immunodeficient child: an insight into the natural evolution of oral polio vaccine. J. Virol. **79:**1062–1070.
6. **Chevaliez, S., A. Szendroi, V. Caro, J. Balanant, S. Guillot, G. Berencsi, and F. Delpeyroux.** 2004. Molecular comparison of echovirus 11 strains circulating in Europe during an epidemic of multisystem hemorrhagic disease of infants indicates that evolution generally occurs by recombination. Virology **325:**56–70.
7. **Equestre, M., D. Genovese, F. Cavalieri, L. Fiore, R. Santoro, and R. Perez-Bercoff.** 1991. Identification of a consistent pattern of mutations in neurovirulent variants derived from the Sabin vaccine strain of poliovirus type 2. J. Virol. **65:**2707–2710.
8. **Estívariz, C. F., M. A. Watkins, D. Handoko, R. Rusipah, J. Deshpande, B. J. Rana, E. Irawan, D. Widhiastuti, M. A. Pallansch, A. Thapa, and S. Imari.** 2008. A large vaccine-derived poliovirus outbreak on Madura Island—Indonesia, 2005. J. Infect. Dis. **197:**347–354.
9. **Georgescu, M. M., M. Tardy-Panit, S. Guillot, R. Crainic, and F. Delpeyroux.** 1995. Mapping of mutations contributing to the temperature sensitivity of the Sabin 1 vaccine strain of poliovirus. J. Virol. **69:**5278–5286.
10. **Horie, H., S. Koike, T. Kurata, Y. Sato-Yoshida, I. Ise, Y. Ota, S. Abe, K. Hioki, H. Kato, C. Taya, et al.** 1994. Transgenic mice carrying the human poliovirus receptor: new animal models for study of poliovirus neurovirulence. J. Virol. **68:**681–688.
11. **Jiang, P., J. A. Faase, H. Toyoda, A. Paul, E. Wimmer, and A. E. Gorbalenya.** 2007. Evidence for emergence of diverse polioviruses from C-cluster coxsackie A viruses and implications for global poliovirus eradication. Proc. Natl. Acad. Sci. USA **104:**9457–9462.
12. **Kew, O., V. Morris-Glasgow, M. Landaverde, C. Burns, J. Shaw, Z. Garib, J. Andre, E. Blackman, C. J. Freeman, J. Jorba, R. Sutter, G. Tambini, L. Venczel, C. Pedreira, F. Laender, H. Shimizu, T. Yoneyama, T. Miyamura, H. van Der Avoort, M. S. Oberste, D. Kilpatrick, S. Cochi, M. Pallansch, and C. de Quadros.** 2002. Outbreak of poliomyelitis in Hispaniola associated with circulating type 1 vaccine-derived poliovirus. Science **296:**356–359.
13. **Kew, O. M., B. K. Nottay, M. H. Hatch, J. H. Nakano, and J. F. Obijeski.** 1981. Multiple genetic changes can occur in the oral poliovaccines upon replication in humans. J. Gen. Virol. **56:**337–347.
14. **Kew, O. M., R. W. Sutter, E. M. de Gourville, W. R. Dowdle, and M. A. Pallansch.** 2005. Vaccine-derived polioviruses and the endgame strategy for global polio eradication. Annu. Rev. Microbiol. **59:**587–635.
15. **Kew, O. M., P. F. Wright, V. I. Agol, F. Delpeyroux, H. Shimizu, N. Nathanson, and M. A. Pallansch.** 2004. Circulating vaccine-derived polioviruses: current state of knowledge. Bull. W. H. O. **82:**16–23.
16. **Lentz, K. N., A. D. Smith, S. C. Geisler, S. Cox, P. Buontempo, A. Skelton, J. DeMartino, E. Rozhon, J. Schwartz, V. Girijavallabhan, J. O'Connell, and E. Arnold.** 1997. Structure of poliovirus type 2 Lansing complexed with antiviral agent SCH48973: comparison of the structural and biological properties of three poliovirus serotypes. Structure **5:**961–978.
17. **Liang, X., Y. Zhang, W. Xu, N. Wen, S. Zuo, L. A. Lee, and J. Yu.** 2006. An outbreak of poliomyelitis caused by type 1 vaccine-derived poliovirus in China. J. Infect. Dis. **194:**545–551.
18. **Macadam, A. J., G. Ferguson, J. Burlison, D. Stone, R. Skuce, J. W. Almond, and P. D. Minor.** 1992. Correlation of RNA secondary structure and attenuation of Sabin vaccine strains of poliovirus in tissue culture. Virology **189:**415–422.
19. **Macadam, A. J., S. R. Pollard, G. Ferguson, R. Skuce, D. Wood, J. W. Almond, and P. D. Minor.** 1993. Genetic basis of attenuation of the Sabin type-2 vaccine strain of poliovirus in primates. Virology **192:**18–26.
20. **Martín, J., G. Dunn, R. Hull, V. Patel, and P. D. Minor.** 2000. Evolution of the Sabin strain of type 3 poliovirus in an immunodeficient patient during the entire 637-day period of virus excretion. J. Virol. **74:**3001–3010.
21. **Martín, J., K. Odoom, G. Tuite, G. Dunn, N. Hopewell, G. Cooper, C. Fitzharris, K. Butler, W. W. Hall, and P. D. Minor.** 2004. Long-term excretion of vaccine-derived poliovirus by a healthy child. J. Virol. **78:**13839–13847.
22. **Merkle, I., M. J. van Ooij, F. J. van Kuppeveld, D. H. Glaudemans, J. M. Galama, A. Henke, R. Zell, and W. J. Melchers.** 2002. Biological significance of a human enterovirus B-specific RNA element in the 3′ nontranslated region. J. Virol. **76:**9900–9909.
23. **Minor, P. D.** 1992. The molecular biology of poliovaccines. J. Gen. Virol. **73:**3065–3077.
24. **Pizzi, M.** 1950. Sampling variation of the fifty percent end-point, determined by the Reed-Muench (Behrens) method. Hum. Biol. **22:**151–190.
25. **Racaniello, V. R.** 2006. One hundred years of poliovirus pathogenesis. Virology **344:**9–16.
26. **Rakoto-Andrianarivelo, M., S. Guillot, J. Iber, J. Balanant, B. Blondel, F. Riquet, J. Martin, O. Kew, B. Randriamanalina, L. Razafinimpiasa, D. Rousset, and F. Delpeyroux.** 2007. Co-circulation and evolution of polioviruses and species C enteroviruses in a district of Madagascar. PLoS Pathog. **3:**e191.
27. **Reed, L. J., and M. Muench.** 1938. A simple method for estimating fifty percent endpoints. Am. J. Hyg. **27:**493–497.
28. **Ren, R., E. G. Moss, and V. R. Racaniello.** 1991. Identification of two determinants that attenuate vaccine-related type 2 poliovirus. J. Virol. **65:** 1377–1382.
29. **Rezapkin, G. V., L. Fan, D. M. Asher, M. R. Fibi, E. M. Dragunsky, and K. M. Chumakov.** 1999. Mutations in Sabin 2 strain of poliovirus and stability of attenuation phenotype. Virology **258:**152–160.
30. **Rieder, E., A. V. Paul, D. W. Kim, J. H. van Boom, and E. Wimmer.** 2000. Genetic and biochemical studies of poliovirus *cis*-acting replication element *cre* in relation to VPg uridylylation. J. Virol. **74:**10371–10380.
31. **Rousset, D., M. Rakoto-Andrianarivelo, R. Razafindratsimandresy, B. Randriamanalina, S. Guillot, J. Balanant, P. Mauclere, and F. Delpeyroux.** 2003.

Recombinant vaccine-derived poliovirus in Madagascar. Emerg. Infect. Dis. **9:**885–887.
32. **Shimizu, H., B. Thorley, F. J. Paladin, K. A. Brussen, V. Stambos, L. Yuen, A. Utama, Y. Tano, M. Arita, H. Yoshida, T. Yoneyama, A. Benegas, S. Roesel, M. Pallansch, O. Kew, and T. Miyamura.** 2004. Circulation of type 1 vaccine-derived poliovirus in the Philippines in 2001. J. Virol. **78:**13512–13521.
33. **Shulman, L. M., Y. Manor, R. Handsher, F. Delpeyroux, M. J. McDonough, T. Halmut, I. Silberstein, J. Alfandari, J. Quay, T. Fisher, J. Robinov, O. M. Kew, R. Crainic, and E. Mendelson.** 2000. Molecular and antigenic characterization of a highly evolved derivative of the type 2 oral poliovaccine strain isolated from sewage in Israel. J. Clin. Microbiol. **38:**3729–3734.
34. **Sutter, R. W., and R. Prevots.** 1994. Vaccine-associated paralytic poliomyelitis among immunodeficient persons. Infect. Med. **11:**426–438.
35. **Tardy-Panit, M., B. Blondel, A. Martin, F. Tekaia, F. Horaud, and F. Delpeyroux.** 1993. A mutation in the RNA polymerase of poliovirus type 1 contributes to attenuation in mice. J. Virol. **67:**4630–4638.
36. **van der Werf, S., J. Bradley, E. Wimmer, F. W. Studier, and J. J. Dunn.** 1986. Synthesis of infectious poliovirus RNA by purified T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83:**2330–2334.
37. **WHO.** 2007. Global update on vaccine-derived polioviruses, January 2006–August 2007. Wkly. Epidemiol. Rec. **82:**337–344.
38. **WHO.** 2006. Vaccine-derived polioviruses—update. Wkly. Epidemiol. Rec. **81:**398–404.
39. **Yang, C. F., T. Naguib, S. J. Yang, E. Nasr, J. Jorba, N. Ahmed, R. Campagnoli, H. van der Avoort, H. Shimizu, T. Yoneyama, T. Miyamura, M. Pallansch, and O. Kew.** 2003. Circulation of endemic type 2 vaccine-derived poliovirus in Egypt from 1983 to 1993. J. Virol. **77:**8366–8377.